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Mutational analysis of the *SOX9* gene in campomelic dysplasia and autosomal sex reversal: lack of genotype/phenotype correlations

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It has previously been shown that, in the heterozygous state, mutations in the *SOX9* gene cause campomelic dysplasia (CD) and the often associated autosomal XY sex reversal. In 12 CD patients, 10 novel mutations and one recurrent mutation were characterized in one *SOX9* allele each, and in one case, no mutation was found. Four missense mutations are all located within the high mobility group (HMG) domain. They either reduce or abolish the DNA-binding ability of the mutant SOX9 proteins. Among the five nonsense and three frameshift mutations identified, two leave the C-terminal transactivation (TA) domain encompassing residues 402–509 of SOX9 partly or almost completely intact. When tested in cell transfection experiments, the recurrent nonsense mutation Y440X, found in two patients who survived for four and more than 9 years, respectively, exhibits some residual transactivation ability. In contrast, a frameshift mutation extending the protein by 70 residues at codon 507, found in a patient who died shortly after birth, showed no transactivation. This is apparently due to instability of the mutant SOX9 protein as demonstrated by Western blotting. Amino acid substitutions and nonsense mutations are found in patients with and without XY sex reversal, indicating that sex reversal in CD is subject to variable penetrance. Finally, none of 18 female patients with XY gonadal dysgenesis (Swyer syndrome) showed an altered *SOX9* banding pattern in SSCP assays, providing evidence that *SOX9* mutations do not usually result in XY sex reversal without skeletal malformations.

INTRODUCTION

Campomelic dysplasia (CD) is a rare, often lethal, dominantly inherited, congenital osteochondrodysplasia, associated with male-to-female autosomal sex reversal in two-thirds of the affected karyotypic males. Prominent features of CD are bowing of femora and tibiae, hypoplastic scapulae, 11 instead of 12 pairs of ribs, Robin sequence, pelvic malformations and bilateral clubfeet. The majority of CD patients die neonatally due to respiratory distress (1,2). By positional cloning in combination with positional candidate information, the *SOX9* gene on chromosome 17q was isolated from the vicinity of breakpoints in CD patients with reciprocal *de novo* translocations (3,4). Proof of *SOX9* being responsible for both CD and XY sex reversal came from the demonstration of *de novo* heterozygous loss-of-function mutations within the *SOX9* coding region in non-translocation CD patients (3–5). Unexpected and still unexplained remains the

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observation that the breakpoints in all six translocation patients studied do not interrupt the *SOX9* gene but map 50 kb or more 5′ to the gene (3,4,6,7). In line with the generalized defect in skeletal development seen in CD patients, the mouse *Sox9* gene has been shown to be expressed in mesenchymal condensations before and during embryonic cartilage deposition, consistent with a primary role for *SOX9/Sox9* in skeletal formation (8).

Like the Y-located testis-determining gene *SRY*, *SOX9* is a member of the *SOX* gene family of transcription factors. SOX proteins share an amino acid sequence identity of 60% or more in their high-mobility group (HMG) domain with the HMG domain present in SRY (9). The HMG domain is an 80 amino acid DNA-binding and bending motif that characterizes, besides the SOX proteins, a whole class of transcription factors (10). Apart from the HMG domain, the 509 residue SOX9 protein contains two additional protein motifs: first, a stretch of 41 residues (residues 339–379) composed solely of proline, glutamine and alanine (PQA motif), the function of which is unknown; secondly, a C-terminal transcription activation domain rich in serine, proline and glutamine, ranging from residues 402–509 (11).

Up to now, a total of 13 *SOX9* mutations have been published (3–5). As these mutations are all heterozygous and appear to cause loss of function of SOX9, CD can be regarded as a haploinsufficiency syndrome. To gain more insight into the mutational spectrum in CD and to see whether or not some genotype/phenotype correlations emerge regarding XY sex reversal, survival or severity of disease, we have extended our previous study to 12 new CD cases with and without XY sex reversal. We have thus identified 10 novel *SOX9* mutations and one recurrent mutation, and have analysed some of these mutations in functional assays. In addition, we have screened DNA samples from patients with XY gonadal dysgenesis (Swyer syndrome) known to have an intact *SRY* gene for mutations in *SOX9*.

RESULTS

Novel and recurrent *SOX9* mutations detected in 12 CD patients

We have screened PCR-derived fragments spanning the complete coding region, the four exon/intron boundaries, and 133 bp from the promoter region of the three-exon *SOX9* gene in DNA from 12 unrelated patients for mutations. Three cases have been screened by the single strand conformation polymorphism (SSCP) assay (12), with two showing an altered banding pattern due to a pathologically relevant mutation as shown by DNA sequence analysis of the relevant PCR product. The third case and the remaining nine cases have been analysed by direct sequencing of PCR products. The karyotypes and sexual phenotypes of the patients studied are listed in Table 1, in addition to the specific mutations detected in all but one of the cases. These mutations, which are all in the heterozygous state, will here be presented in detail.

A W86X (TGG→TAG) nonsense mutation was identified proximal to the HMG box in patient M.R., removing all functional domains of SOX9. A sister of this patient also died from CD shortly after birth. Whereas DNA from this sister was unavailable, leukocyte DNA from a healthy sister, from both parents as well as amniocyte DNA from an actual pregnancy proved negative for the W86X mutation. Germ-line mosaicism in one of the parents is the likely cause for the recurrence of CD in this family. Another nonsense mutation, Q117X (CAG→TAG), was identified in patient J.N., who survived for 12 years. This mutation occurs in the first third of the HMG box, leading to a truncated protein lacking 393 of 509 amino acid (aa) residues. Therefore, the resulting protein should be deficient in most, if not all, of its functional domains. DNA of both parents proved negative for the mutation.

Missense mutations resulting in reduction or loss of DNA binding of the mutant SOX9 protein (see below) were detected in four patients. A P108L (CCC→CTC) and a W143R (TGG→CGG) mutation were found in patients A.S. and K.D., respectively. Patient M.P. with a R152P (CGG→CCG) mutation shows acampomelic CD. A P170R (CCG→CGG) mutation affecting a highly conserved proline residue was detected in male patient T.L. The *de novo* nature of the latter two mutations was inferred from their absence in parental DNA.

A *de novo* deletion of 10 bp [nucleotide (nt) position 1192–1201; numbering according to ref. 3], resulting in a codon encoding a mutant serine residue at aa position 277 followed by an *opal* stop codon, was detected in the *SOX9* gene of patient L-M.A.. Another 43 bp deletion (nt position 1442–1484) results in a frameshift creating 11 mutant aa residues following residue 356 in patient F.

Table 1. *SOX9* mutations in patients with campomelic dysplasia identified in this study

Patient	Karyotype and gender	Nucleotide (nt) position and mutation	Amino acid position and type of mutation	Survival time
M.R.	46, XX, F	nt 629, TGG→TAG	W86X, nonsense, *de novo*	12 days
A.S.	46, XY, F	nt 695, CCC→CTC	P108L, missense	6 months
J.N.	46, XY, F	nt 721, CAG→TAG	Q117X, nonsense, *de novo*	alive at 12 years
K.D.	46, XY, F	nt 799, TGG→CGG	W143R, missense	5½ months
M.P.	46, XX, F	nt 827, CGG→CCG	R152P, missense, *de novo*	2 days
T.L.	46, XY, inv(9), M	nt 881, CCG→CGG	P170R, missense, *de novo*	1 month
L–M.A.	46, XX, F	del(1192–1201)	frameshift at codon 277	10 weeks
F	46, XY, F	del(1442–1484)	frameshift at codon 357	2 days
M.J.	46, XY, F	nt 1495, CAG→TAG	Q375X, nonsense, *de novo*	7 weeks
P.G.	46, XY, M	nt 1570, GAG→TAG	E400X, nonsense	10 weeks
N.Z.	46, XX, F	nt 1692, TAC→TAG	Y440X, nonsense	alive at 11 years
R.R.	46, XY, M	no mutation found	no mutation found	alive at 5½ years

Numbering of nucleotide and amino acid positions are according to ref. (3). For the position of the mutations relative to the *SOX9* gene structure, see Figure 3. F, female; M, male.

A nonsense mutation within codon 375 (Q375X, CAG→TAG) is localized in the distal part of the PQA-encoding region of patient M.J., leaving 36 of 41 amino acid residues of this domain intact. Both parents are homozygous for the wild-type allele, proving that the Q375X mutation is *de novo*. A further nonsense mutation at codon 400 (E400X; GAG→TAG) results in skeletal malformations, but not in XY sex reversal in patient P.G. In this case, the HMG domain remains intact, whereas the transactivation domain at the C-terminus of the protein is missing.

Previously, we reported a *de novo* nonsense mutation within codon 440 (Y440X; TAC→TAG) in patient S.P. (3). We have now identified the same Y440X mutation in another patient, N.Z. This stop codon mutation results in a truncated protein missing 70 aa residues at its C-terminus. S.P., a sex reversed XY female, survived for 4 years (13), whereas N.Z., a karyotypic and phenotypic female, is still alive at the age of 10. Maternal, but not paternal DNA, could be tested and proved negative for the mutation.

Finally, in CD patient R.R., as in patients H.H. and J.M. analysed previously (3), no mutation could be detected by sequencing of the open reading frame (ORF) and promoter region (from start codon to nt –133) of *SOX9*. To see whether or not one of the *SOX9* alleles may have been deleted, a Southern blot prepared from *Eco*RI-digested genomic DNA of all of the three CD cases was hybridized simultaneously with the *SOX9* cDNA probe 4.1 (3) detecting a 6 kb *Eco*RI fragment, and with cDNA probe pUCTAT2.16 from the human tyrosine aminotransferase (TAT) gene (14) detecting a 5.8 kb *Eco*RI fragment from chromosome 16. The signal intensity of the *SOX9*-specific fragment relative to the *TAT* control fragment was the same in the CD cases and in controls (not shown), indicating that both *SOX9* alleles are present. Patients R.R. and J.M. are heterozygous for the G/C polymorphism at nt 879 (3). This observation also confirms presence of both alleles.

Mutant SOX9 proteins with reduced and abolished transactivation activity

As mentioned above, patients S.P. (3) and N.Z. (Table 1) share the recurrent stop codon mutation Y440X that truncates the C-terminal transactivation (TA) domain spanning residues 402–509 (11). Both patients survived the neonatal period by years. In contrast, patient GM04329 (5) with a frameshift at codon 507 leaving all of SOX9 including the TA domain essentially intact, but extending the protein by 70 mutant residues, died shortly after birth. We have elucidated the mutation in the *SOX9* gene of this patient, a girl with karyotype 46, XX, as a duplication of the ACTC motif at nt position 1891. In order to understand the cause for the differences in the state of health of the patients, we grafted residues 402–439 of the TA domain of SOX9 to the DNA binding domain of yeast transcription factor GAL4 (Fig. 1a) and measured transcriptional activation of a GAL4-dependent CAT reporter plasmid cotransfected in COS-7 cells, as previously described (11). As shown in Figure 1b, the resulting fusion protein GAL4-SOX9/402–439 shows threefold CAT-induction, whereas the full length SOX9 grafted to the GAL4 domain (GAL4-SOX9/1–509) gives 12-fold CAT-induction compared with the value obtained for the GAL4 domain alone. These data identify a residual transcription activation potential for residues 402–439 of SOX9. These residues are still present in the mutationally truncated proteins from patients S.P. and N.Z. As also shown in Figure 1, grafting of the mutationally extended SOX9 protein of patient GM04329 to the GAL4 domain results in fusion protein GAL4-SOX9/1–577, which shows no increase in CAT-induction. To check for correct protein size and stability of the GAL4 fusion proteins, Western blot analysis was performed using a GAL4-specific polyclonal antibody. As illustrated in Figure 1c, GAL4-SOX9/1–509 and GAL4-SOX9/402–439 show correct size and stability as does the GAL4 domain alone. By contrast, fusion protein GAL4-SOX9/1–577 shows correct size but strongly reduced amounts of protein. This result was obtained for nuclear extracts from independent transfection experiments. It appears that the extension of SOX9 by 70 residues creates an unstable mRNA or protein, thus explaining the lack of transactivation observed in Figure 1b.

Amino acid substitutions in the HMG domain of SOX9 lead to reduced or abolished DNA binding

The point mutations causing the aa substitutions P108L, W143R, R152P and P170R found in four CD patients (see above) were introduced by site-directed mutagenesis into plasmid pCDNA3-SOX9-HMG (see Materials and Methods) containing the wild-type *SOX9* HMG box. Proteins were expressed from the wild-type and mutant constructs using a coupled *in vitro* transcription/translation system. They were subsequently tested in a gel retardation assay to see whether the mutant SOX9 HMG domains show altered DNA binding abilities. The wild-type SOX9 HMG domain readily binds oligonucleotide A containing the high-affinity binding site AACAAT for murine (15) and human SRY (16) (Fig. 2, lane 3), but not the control oligonucleotide C (Fig. 2, lane 2) with a mutant binding sequence. Of the four mutant SOX9 HMG-domains, P108L and W143R completely fail to bind oligonucleotide A (Fig. 2, lanes 5 and 6), while R152P and P170R show reduced DNA binding, forming complexes that reproducibly move faster (Fig. 2, lane 4) or slower (Fig. 2, lane 7) than the complexes formed by the wild-type HMG domain. In these experiments, an unspecific complex is formed by proteins present in the wheat germ extract (Fig. 2, lane 1).

Absence of *SOX9* mutations in sex reversed patients with XY gonadal dysgenesis

To find out whether mutations in *SOX9* may also result in XY gonadal dysgenesis (Swyer syndrome), as do mutations in the related testis determining gene *SRY*, we examined 91% of the *SOX9* ORF and the four exon/intron boundaries in a total of 18 patients with Swyer syndrome by SSCP analysis. All cases have proved negative for mutations in the *SRY* ORF in SSCP assays and by DNA sequencing (17,18, and unpublished data). In none of the cases could an altered *SOX9* banding pattern be detected (data not shown), providing evidence that *SOX9* mutations do not usually result in XY sex reversal without skeletal malformations.

DISCUSSION

Here we present 10 novel mutations and one recurrent mutation in *SOX9*, the gene responsible for both CD and autosomal XY sex reversal. Including 13 *SOX9* mutations previously reported (3–5), altogether 24 *SOX9* mutations are now known (see Fig. 3). Nine of them are located within the HMG box, which encodes the DNA binding domain of the protein. These nine mutations include all six amino acid substitutions described so far in CD. As the amino acid sequences of SOX9 proteins from human (3,4), mouse (8)

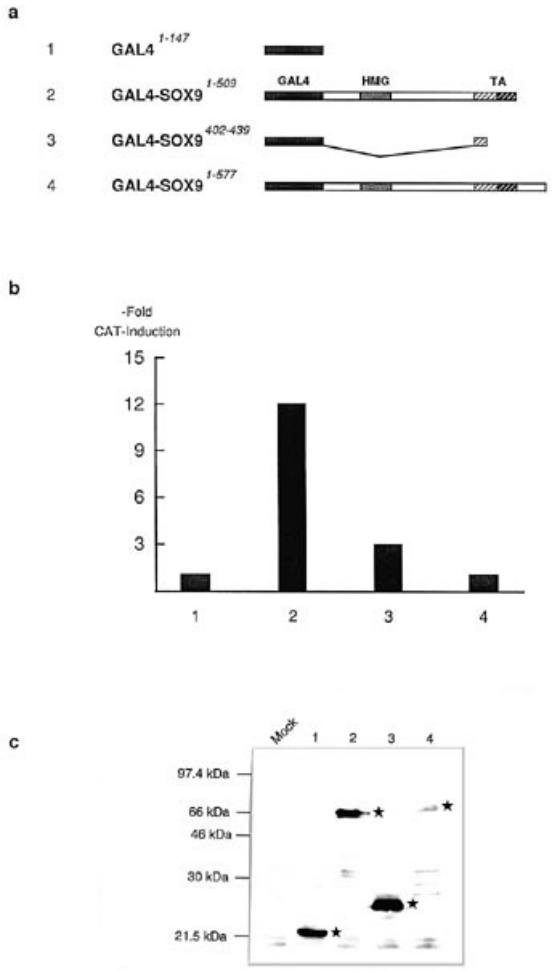

Figure 1. Effects of nonsense mutation Y440X and of frameshift mutation (+ACTC) at codon 507 on the transactivation function of SOX9. (**a**) Constructs used. 1, DNA-binding domain of yeast GAL4 (amino acids 1–147, given as filled bar). 2, wild-type SOX9 fused to GAL4. 3, part of the TA domain retained in Y440X mutated SOX9 fused to GAL4. 4, mutant SOX9 with C-terminal extension due to (ACTC)-duplication at codon 507 and resulting frameshift fused to GAL4. HMG, high mobility group domain; TA, transactivation domain. (**b**) Results of CAT assays. Constructs 1–4 shown in (a) were co-transfected with GAL4-dependent CAT reporter plasmid 17mertkCAT (29) into COS-7 cells and were tested for stimulation of CAT activity. CAT values obtained for each construct are given relative to the value obtained for GAL4 alone, set at 1. Values were averaged from three independent experiments, with standard deviations below 20%. (**c**) Western blot. Nuclear proteins from untransfected 293 cells (mock) or from 293 cells transfected with constructs 1–4 shown in (a) were detected by a GAL4-specific antibody following Western blotting. Proteins of the expected size are visible in lanes 1–4 and are marked by asterisks. Note the reduced amount of fusion protein in lane 4, compared with lanes 1–3 showing higher and roughly equal amounts of protein. Position of size markers (Rainbow marker, Amersham) are given at left.

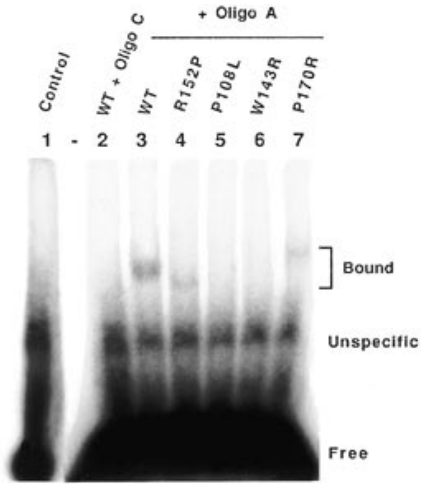

Figure 2. Amino acid substitutions in the HMG domain of SOX9 reduce or abolish DNA binding. Gel retardation assays were performed with equal amounts of protein obtained from a coupled wheat germ *in vitro* transcription/translation reaction, together with oligonucleotide probes A (oligo A) or C (oligo C) containing the target sequence AACAAT or the nontarget sequence AACCCA, respectively (18). Lane 1, protein synthesis reaction without added template, together with oligo A. Lanes 2–7, wild-type or mutant SOX9 HMG domains, as indicated. Position of free and bound probe is indicated at right, as is an unspecific complex formed by proteins in the wheat germ extract.

and chicken (GenBank accession no. U12533) are highly conserved over the entire N-terminal half of the protein, including the HMG domain, and within the C-terminal TA domain (11), it appears that the amino acid sequence of the HMG domain is particularly critical for correct function.

All four patients carrying the missense mutations P108L, W143R, R152P and P170R died within 6 months after birth. We have analysed these mutations with respect to their effects on DNA binding of the resulting mutant HMG domains. The P108L and W143R mutations cause complete loss of binding, while the HMG domains with the R152P and P170R mutations show reduced DNA binding (Fig. 2). The observation that the latter two mutations result in protein/DNA complexes moving slower or faster than the wild-type complexes may indicate that these amino acid complexes also result in an altered DNA bending angle, as has been demonstrated for one amino acid substitution in the HMG domain of SRY found in an XY female with gonadal dysgenesis (19). However, SOX9 has not yet been shown to be a DNA binding protein.

The W143R mutation affects the most conserved amino acid residue among the HMG domain proteins. This residue is present at a corresponding position in 39 of 44 members of this protein family and is present in all SOX proteins (20). It is not surprising, therefore, that the W143R mutation shows complete loss of DNA binding. Interestingly, replacement of the corresponding tryptophan residue by arginine in the HMG domain of the HMG1 protein results in an altered protein structure and affects many, but not all, of its DNA binding properties (21,22).

Of all *SOX9* mutations that are not missense mutations, only the recurrent nonsense mutation Y440X found in patients S.P. (3) and N.Z. (Table 1) and the frameshift mutation at codon 507 in patient GM04329 (5) leave part or almost all of the TA domain intact (Fig. 3). We have demonstrated in functional transfection assays that the truncated SOX9 protein resulting from the Y440X mutation retains some transactivation function, while the mutant SOX9 protein resulting from the codon 507 frameshift shows no transactivation, probably due to instability of the protein, or its mRNA (Fig. 1). These findings correlate with the clinical course of the patients involved, as S.P. and N.Z. survived the neonatal period, whereas patient GM04329 died shortly after birth. The other nonsense and frameshift mutations described so far, and the two splice mutations, result in SOX9 proteins that entirely lack

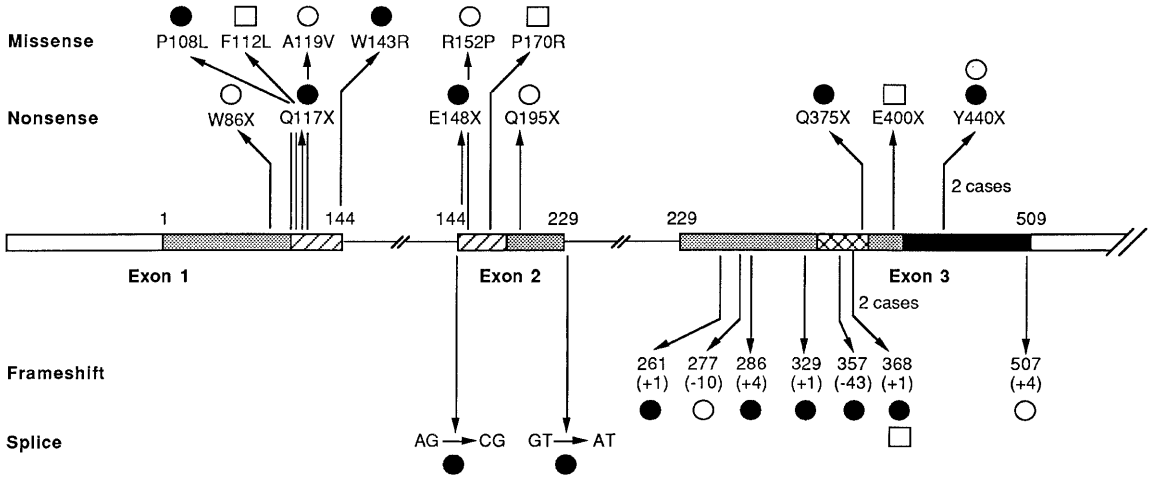

Figure 3. Summary of presently known *SOX9* mutations in campomelic dysplasia and autosomal XY sex reversal. Exons 1–3 of *SOX9* are drawn to scale, with exon 3 being truncated. Coding sequences are indicated by gray, stippled (HMG domain), cross-hatched (PQA motif) or black boxes (transactivation domain), untranslated regions by white boxes. Numbers indicate codons or amino acid residues, given as single letter code. The nature of the mutations is indicated at left. The number of bases inserted or deleted in frameshift mutations is given in brackets. Mutations found in XY and XX females are marked by filled and open circles, respectively, while mutations present in XY males are indicated by open squares. Data are from this report (Table 1); ref. 3 (E148X, Y440X, 329(+1), splice donor GT→AT); ref. 4 (Q195X, 261(+1), 286(+1); and ref. 5 (F112L, A119V, 368(+1), 368(+1), 507(+4), splice acceptor AG→CG).

the TA domain; additionally, some mutant SOX9 proteins lack part or all of the HMG domain (Fig. 3). As might be expected, most of the patients having such severely impaired SOX9 proteins died in the neonatal period. This is the case for most of the patients previously described (3–5), and for five of the six such patients with nonsense mutations W86X, Q375X and E400X, and those with frameshift mutations at codons 277 and 357 (Table 1, see also patient reports). However, patient J.N. carrying the nonsense mutation Q117X at the N-terminus of the HMG domain is now doing well at the age of 12 years.

Four *SOX9* mutations in phenotypic male CD patients have now been reported. Two are amino acid substitutions in the HMG domain, F112L (5) and P170R (Table 1) (see Fig. 3). Whereas the F112L mutation has not been tested functionally, the P170R mutation in patient T.L. has been shown to retain some DNA binding ability (Fig. 2). While it is tempting to correlate this with the lack of sex reversal seen in this patient, we are reluctant to do so in view of the fact that residual DNA binding has also been documented for some amino acid substitutions in the HMG domain of SRY found in XY females with gonadal dysgenesis (18,19,23,24). The E400X mutation in the male patient P.G. described here results in a truncated protein completely lacking the transactivating domain at its C-terminus (11). As nonsense mutations flanking codon 400 on either side are found in sex-reversed XY females (Q375X and Y440X; Table 1), no correlation between the position of a stop codon in *SOX9* and the sexual phenotype is apparent. Finally, a *SOX9* mutation described in a male CD patient is a single A insertion in codon 368 (5), but an identical mutation was also found in a sex reversed XY female (5) (Fig. 3), providing a particularly clear example that XY sex reversal in *SOX9* is subject to variable penetrance. None of the four phenotypic and karyotypic male patients with known *SOX9* mutations survived beyond the neonatal period. However, a case of a 17 year old male long-term-survivor is documented (1,25).

Although cases of CD without male to female sex reversal are common, no case of sex reversal without skeletal malformations caused by a *SOX9* mutation has been reported to date. As both *SRY* and *SOX9* act within the sex determination/differentation pathway, one could assume that *SOX9* mutations may also cause gonadal dysgenesis and XY sex reversal (Swyer syndrome), as do mutations in *SRY*. However, we detected no *SOX9* mutations in 18 patients with Swyer syndrome carrying an intact *SRY* gene. As only 91% of the gene has been analysed by SSCP in these 18 cases, we cannot completely rule out that *SOX9* mutations may occasionally cause Swyer syndrome in other patients.

In patient R.R. (Table 1), as in three patients previously studied (3,5), no *SOX9* mutations and no chromosomal rearrangements could be detected. In all CD cases with chromosomal aberrations analysed so far, the translocation breakpoints are located at remarkable distances of 50 kb to more than 130 kb 5′ from *SOX9*, providing evidence for an extended control region of the *SOX9* gene (3,4,6,7). In the four CD cases with no detectable *SOX9* structural gene mutation, the mutations may be located within the putative far upstream regulatory element(s) affected by the translocations. Alternatively, the mutations may reside within intronic sequences of the *SOX9* gene, or may affect the putative expressed sequence 5′ to *SOX9* described by Ninomiya *et al.* (26).

As discussed above, a major conclusion from this and from previous reports is the lack of correlation between the type and position of mutation within the gene with the resulting phenotype. It appears that both XY sex reversal and disease severity are a matter of penetrance of a mutation rather than the result of a specific type of mutation: a patient may have a nonsense mutation removing 80% of the protein and survive for several years, as in patient J.N. with the Q117X mutation, while nonsense or frameshift mutations leaving a much larger segment of SOX9 intact are found in patients who died in the neonatal period. The only tentative genotype/phenotype correlation we can formulate concerns the Y440X mutation, where the retention of some transactivation potential may be causally related with the fact that the two unrelated patients carrying this mutation survived for several years. Future mutational studies of *SOX9* in CD patients

may show whether this correlation linking residual trans-activating activity of a mutant SOX9 protein to an increased survival rate will hold.

MATERIALS AND METHODS

Patient reports

M.R. (Vienna) is the third child of non-consanguineous, healthy parents. One of her two sisters also suffered from CD and died shortly after birth. M.R. showed severe hypoplasia of the scapulae, bowing and shortening of femora and tibiae, clubfeet and low-set ears. She died after 12 days from respiratory insufficiency. Autopsy revealed symmetric hypoplasia of olfactory tracts and bulbs. Karyotype was 46, XX.

Patient A.S. (Munich) is the third child of non-consanguineous parents. She showed postnatal dyspnoea. Morphologic examination revealed flat face, low-set ears, cleft palate, shortened arms, bowing of femora and tibiae, clubfeet, hypoplastic scapulae, stenosis of the trachea and external female genitalia. She died 6 months after birth. Karyotype was 46, XY.

Patient J.N. (Loverval): Clinical details have been reported by Gillerot *et al.* (referred as 'patient 2') (27). The external genitalia are those of a normal female. Owing to her orthopedic problems, she is now wheelchair bound. For her hypoacousia—characteristic of the syndrome—she received an external amplifier and markedly progressed in her psychomotor development. She is mildly retarded, has a high-pitched voice and is now doing well with good social capabilities. Her weight is 17.5 kg and her height is of 102 cm (–6 SD) at the age of 12 years. Karyotype is 46, XY.

Patient K.D. (Leuven): First child of unrelated, healthy parents. Presented with a large head, low-set ears, broad nasal bridge, broad alveolar ridges, bifid uvula, bowed lower limbs with pretibial skin 'dimples', and bilateral clubfeet. There was hypoplasia of cervical (C5–C7) and dorsal (D1 and D2) vertebrae, hypoplastic scapulae, diaphyseal angulation of femora, tibiae and fibulae and short metacarpals in the fourth and fifth fingers. At 5½ months the patient died of an apneic attack. Female type gonads were reduced to fibrous streaks. Karyotype was 46, XY.

Patient M.P. (Aarhus): This case of a patient with acampomelic CD has been published by Friedrich *et al.* (28). She died 2 days after birth. Karyotype was 46, XX.

Patient T.L. (Suhl): Second child of nonconsanguineous parents. His mother has bilateral hip dysplasia, his brother had several hip operations. T.L. showed classical signs of CD including Robin sequence, low-set ears, bowing of tibiae, hypoplastic scapulae and bowed lower limbs. External genitalia were of normal male type. The neonate died 4 weeks after birth. Karyotype was 46, XY, inv(9) (p11q12).

Patient L-M.A. (Berlin) was the first child of healthy, unrelated parents. She showed typical signs of CD: bowing of lower limbs, clubfeet, hypoplasia of scapulae and fibulae, cleft palate, hypoplasia of mandibula and tracheal/bronchial instability. She died at 10 weeks of age. Karyotype was 46, XX.

Patient F. This case of a sex-reversed female has been published by Ebensperger *et al.* (13). Karyotype was 46, XY.

Patient M.J. (Erlangen) was the first child of healthy, unrelated parents. Her CD included short neck, hypoplastic mandible, a large anterior fontanel, flat facies, low-set dysplastic ears, cleft palate, bowed and shortened arms and legs, praetibial skin 'dimples' and normal female external genitalia. Radiography showed hypoplasia of the cervical spine, the scapulae and the pelvis, bowing of femora and tibiae, hypoplastic fibulae and short metacarpal bones. Pyelography revealed low positioned kidneys and mild dilatation of calices. She died after 7 weeks from respiratory distress. Karyotype was 46, XY.

Patient P.G. (Chemnitz) was the first child of healthy parents. Disproportionate short stature, macrocephaly and malformation of the lower limbs were recognized by ultrasound examination during early pregnancy. Postnatal examination confirmed macrocephaly with prominent occiput, Robin sequence with cleft palate and micrognathia, dislocation of the hips, normal male external genitalia, short anteriorly bowed legs and talipes equinovarus. Radiographic signs were narrow vertebral bodies, 11 pairs of ribs, hypoplasia of the scapulae and pelvic bones, shortened upper limbs, antecurvation and varus deformity of the femora and hypoplastic fibulae. He died after 10 weeks due to respiratory distress. Karyotype was 46, XY.

Patient N.Z. (Zürich) was born as first child of healthy parents. At 28 weeks of pregnancy, ultrasound indicated fetal growth retardation. There was bowing of the femora, a very small mandible, prominent forehead and prominent lower occiput, small ears, narrow external ear canals, Robin sequence, bilateral clubfeet, short limbs, especially rhizomelic brachymelia. Feeding was through tube for the first 7 months. During the first 2 years of life, she suffered from recurrent otitis. At 2 years a conductive hearing loss was found bilaterally, more severe on the left. At the age of 8 years, she had severe kyphoscoliosis with gibbus, pedes adducti, impaired hearing and distinct developmental and mental delay. She was placed in a remedial school. First steps without support at 18 months, first words at 2½ years. She is now 11 years of age. Karyotype is 46, XX.

Patient GM04329 (see section 'Mutant SOX9 proteins with reduced and abolished transactivation activity'): This case of a female with karyotype 46, XX has also been studied by Kwok *et al.* (5). The patient died shortly after birth. Fibroblasts were obtained from the Coriell Institute, Camden, USA.

Patient R.R. (Zurich): This mildly affected boy is still alive at the age of 5½ years. He shows hip dysplasia, micrognathia, mildly bowed femora, humeri and radii, 11 pairs of ribs, macrocephaly, and stenosis of the trachea. Scapulae were normal. He shows no Robin sequence and no clubfeet. Karyotype is 46, XY.

PCR and sequence analysis

The three exons and the exon/intron boundaries of the *SOX9* gene were amplified by PCR using the primers and conditions essentially as decribed (3). For the third exon, an additional primer pair: E3-F4: 5′-CCC GGA GGG TGC CTA AGA CTA-3′, E3-R1: 5′-GAT GTG CGT TCG CTG GGA CTG-3′, with a resulting product of 583 bp, was used. DNA sequence analysis of gel-purified PCR products was as described (3).

SSCP

Seven and a half µl PCR product and 7.5 µl denaturing solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) were loaded on a 6% acrylamide/bisacrylamide (29:1) gel run in 45 mM Tris-borate buffer, pH 7.8, 1 mM EDTA. If necessary, PCR products were digested by appropriate restriction enzymes to yield fragment sizes below 300 bp. Electrophoresis was carried out at 4°C, 200 V for 15 h. Gels were fixed for 20 min in 10% ethanol, 0.5% acetic acid and stained for 25 min in a 0.17% (w/v) silver nitrate solution. Stained gels were

rinsed in water for 3 min and developed in 3% NaOH/0.1% formaldehyde solution for 10–15 min. Development was stopped by soaking the gel in fixation solution.

Constructs and CAT assays

Plasmids pABgal (29), designated as GAL4, and GAL4-SOX9/1–509 were described (11). For constructing GAL4-SOX9/1–577, a portion of the *SOX9* gene derived from patient GM04329 was amplified using primers E3-F-PII: 5′-GAC GGA GCA GCT GAG CCC CAG-3′ spanning the *Pvu*II site (underlined) in the third exon of *SOX9*, and E3-R-BI: 5′-CAA GCG TGG AG GAT CCT GGA GA-3′, introducing a *Bam*HI site (underlined) 319 nt 3′ from the wild-type stop codon and 119 nt 3′ from the stop codon of the mutant allele. The *Pvu*II/*Bam*HI portion of GAL4-SOX9/1–509 was then replaced by the 647 bp *Pvu*II/*Bam*HI-digested PCR product resulting in GAL4-SOX9/1–577. In-frame insertion, presence of the expected mutation and lack of mutations created by *Taq* polymerase was monitored by sequencing the resulting clones.

GAL4-SOX9/402–439 was constructed by amplifying a fragment from the wild-type *SOX9* gene using primers SOX9-TA2: 5′-GC GAC CCG GGG CTG AGC CCC AGC CAC TAC-3′ and SOX9-C: 5′-GC GGA TCC CTA CTG TGA GCG GGT GAT GG-3′; introduced *Bam*HI and *Sma*I sites are underlined. The resulting PCR product was cloned into the TA cloning vector pCRII (Invitrogen), resulting in clone pCRII-SOX9/402–439, and verified by sequencing. The 1.6 kb *Sma*I/*Bam*HI fragment of GAL4-SOX9/1–509 containing the whole *SOX9* ORF was then replaced by the 121 bp *Sma*I/*Bam*HI fragment from pCRII-SOX9/402–439 resulting in GAL4-SOX9/402–439.

Expression vector (0.25 pmol) was co-transfected into 1×10^6 COS-7 cells together with 1 pmol CAT reporter construct 17mertkCAT (29). CAT assay was essentially as described (30).

Constructs, mutagenesis and gel retardation assays

The HMG box of *SOX9* was amplified by PCR from cDNA clone 4.1 (3). The 5′-primer, 5′-G CGG GAT CCG CCA GCC ATG CAC GTC AAG CGG CCC ATG AAC-3′, contained a new start codon (underlined) preceded by a Kozak sequence and followed by 21 nucleotides from the 5′ end of the HMG box, and a *Bam*HI site for cloning. The 3′ primer, 5′-CCG GAA TTC TCT GCC TCC GCC TGC CC-3′, spanned 18 nucleotides corresponding to codons 185–190, followed by an *Eco*RI cloning site. Following digestion with *Bam*HI and *Eco*RI, the PCR product was inserted in appropriately cut expression vector pcDNA3, resulting in pcDNA3-SOX9-HMG, and verified by sequencing. An inframe stop codon was provided by adjacent sequences of pcDNA3. Mutations P108L, W143R, R152P and P170R (see Table 1) were introduced into pcDNA3-SOX9-HMG using the QuikChange site-directed mutagenesis kit (Stratagene). Proteins were expressed from the constructs using the TNT T7 coupled wheat germ extract system (Promega) in the presence of ^{35}S-methionine. Correct size of proteins was determined by comparison with the low range prestained SDS–PAGE standard (Bio-Rad). Equal amounts of proteins (as determined by quantification of the ^{35}S-methionine on a SDS–polyacrylamide gel) were used for gel retardation analysis, together with 100 ng of oligonucleotide probe A or C (18) ^{32}P-labeled using T7 polynucleotide kinase. Incubation of probe with protein, electrophoresis and autoradiography were essentially as described (18).

Western blotting

Approximately 10^5 human 293 cells, transfected by the calcium phosphate method (31), were harvested after 36 h, lysed in 1×SDS buffer, and proteins were separated on a reducing 10% SDS–polyacrylamide gel. Subsequently, proteins were transferred on to a PVDF-membrane (Biorad) in a semi-dry-blot apparatus (Schleicher and Schüll). The detection of GAL4 fusion proteins was performed by first washing the membrane twice in TBST (10 mM Tris/HCl, pH 8, 150 mM NaCl, 0.05% Tween 20) and a subsequent incubation in TBST containing 5% non-fat dry milk powder for 1 h. The membrane was then incubated in a small volume of TBST, containing an 1:500 dilution of an antibody directed against the DNA-binding domain of the GAL4 protein. After 4 h incubation at room temperature, the membrane was washed eight times in TBST and incubated for another hour in TBST containing a 1:3000 dilution of the anti-rabbit second antibody coupled to horseradish peroxidase (Biorad). After extensive washing the bound antibodies were detected using the ECL-system (Amersham). Protein molecular weight was determined using the ‘rainbow marker’ (Amersham).

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ABBREVIATIONS

aa, amino acid(s); CAT, chloramphenicol acetyltransferase; CD, campomelic dysplasia; HMG, high-mobility group (box, domain); nt, nucleotide(s); kb, kilobase pairs; ORF, open reading frame; PCR, polymerase chain reaction; PQA, proline-glutamine-alanine (motif); SSCP, single strand conformation polymorphism; TA, transcription activation (domain); TAT, tyrosine aminotransferase.

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